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Review

The missing links to link ubiquitin: Methods for the enzymatic production of polyubiquitin chains

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ABSTRACT

Attachment of ubiquitin (Ub) as monoUb and polyUb chains of different lengths and linkages to proteins plays a dominant role in very different regulatory mechanisms. Therefore, the study of polyUb chains has assumed a central interest in biochemistry and structural biology. An essential step necessary to allow *in vitro* biochemical and structural studies of polyUbs is the production of their chains in high quantities and purity. This is not always an easy task and can be achieved both enzymatically and chemically. Previous reviews have covered chemical cross-linking exhaustively. In this review, we concentrate on the different approaches developed so far for the enzymatic production of different Ub chains. These strategies permit a certain flexibility in the production of chains with various linkages and lengths. We critically describe the available methods and comment on advantages and limitations. It is clear that the field is mature to study most of the possible links, but some more work needs to be done to complete the picture and to exploit the current methodologies for understanding in full the Ub code.

Contents

Abbreviations: Ub, ubiquitin; ATP, adenosine triphosphate; YUH1, yeast protease Ub hydrolase-1; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; DUB, deubiquitylase; UBD, Ub-binding domain; Boc, butyloxycarbonyl.

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Ubiquitination is one of the most important post-translational modifications because it is involved in several essential cellular pathways [1–3]. It arises from the ability of ubiquitin (Ub) to cross-link either with another Ub or with different proteins and form a covalent bond between the C-terminal carboxyl group of glycine 76 of Ub and the ε-amino group of a target protein lysine side chain (Fig. 1A). When cross-linking occurs between two Ub molecules conventionally, during the formation of an isopeptide bond, the proximal Ub is the one linked through the lysine residue, whereas the Ub linked by the C-terminal glycine is called distal Ub. Because Ub has seven lysines, it can form eight structurally and functionally distinct chain types in which Ub moieties are linked via one of the seven Lys residues or the amino terminus [2]. This leads to chains with any of the following linkages: Met1, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63 [4] (Fig. 1B). Chains with mixed linkages and branched chains have also been identified *in vivo* [5,6]. Different linkages have different functional specificities [7]. Lys48- and Lys63-linked polyUb chains are linked to protein degradation [8] and cellular signaling [9], whereas the role of the remaining Ub chain types, often indicated as "atypical" chains, is less understood [10]. Lys11 and Met1 linkages seem to be involved in cell cycle regulation and nuclear factor-κB activation, respectively [11,12]. Unanchored Ub chains cause the activation of protein kinases as TAK1 and IKK [13,14]. It is likely that other new roles in cells will be unveiled.

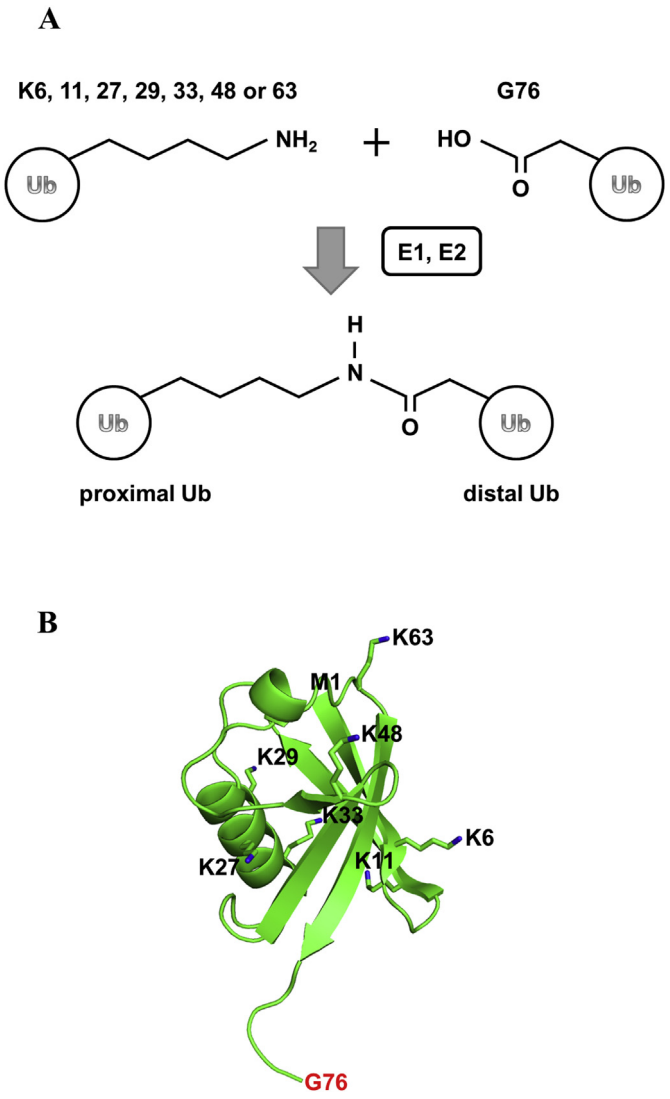

Fig.1. (A) Scheme of the formation of an isopeptide bond between two Ub molecules. (B) Tridimensional structure of ubiquitin (PDB code 1UBQ). All seven lysines, plus the N-terminal methionine and the C-terminal glycine, are labeled.

Ubiquitination is catalyzed *in vivo* by a three-step enzymatic cascade. An E1 Ub activating enzyme, in the presence of adenosine triphosphate (ATP), transfers Ub to the active site cysteine of a E2 Ub conjugating enzyme [15–17]. E2 then directly transfers Ub to a lysine of the substrate, often with the help of an E3 Ub ligase [18], or performs a transthiolation by transferring Ub to the active site cysteine of an E3 enzyme, which is responsible for the ubiquitination of the substrate [19]. The presence of a wide set of specific E2 and E3 enzymes accounts for the fine tuning and complexity of Ub signaling [20].

To unveil the molecular mechanisms responsible for the *in vivo* effects of protein ubiquitination, it is necessary to produce large quantities of pure samples of ubiquitinated proteins and Ub chains for a biochemical, biophysical, and structural characterization. Ubiquitination has been obtained either by chemical synthesis of suitable functionalized precursors or by enzymatic assembly. Several strategies have been proposed for the enzymatic production of monoubiquitinated proteins at high yields [21]. A recent article also described a new method to obtain polyubiquitinated proteins [22].

Here, we concentrate on the problem of producing polyUb chains. DiUbs of all eight possible types are commercially available (Life Sensors, Boston Biochem, Enzo Life Sciences). Longer K48-, K63-, and K11-linked and linear chains are also available, but the requirement of milligrams of sample makes the elevate cost prohibitive. Luckily, production of Ub chains is relatively less complex than synthetizing ubiquitinated proteins; Ub is a very stable protein that can be easily refolded, allowing the possibility to work in denaturing conditions for the chemical synthesis of the chains. Alternatively, the specific enzymes for the formation of most Ub chain types are also known and well characterized, allowing an enzymatic procedure.

Several chemical or enzymatic approaches have been developed. Chemical methods are generally based on the preparation of reactive precursors that are ligated by native chemical ligation. Because these non-enzymatic methods have been amply reviewed before, we refer the reader to previous publications for details [23–25]. In this review, we focus on the strategies developed for the enzymatic synthesis of polyUb chains of selected lengths and linkages. The main two approaches developed differ for the Ub substrate used, either Ub mutants or wild-type Ub. We describe the methods developed so far and provide examples of applications for different chain types.

Production of polyUb chains using Ub mutants

A rather convenient enzymatic method for the production of K48-linked Ub chains of selected length was reported in 1997 by Pickart and coworkers [26]. The method was later extended to K63-linked chains, and detailed protocols were published [27,28]. It relies on the ability of an E2 Ub conjugating enzyme to form a specific type of isopeptide bond between two Ub molecules. An E1 enzyme activates Ub for the reaction, and the specific E2 catalyzes the conjugation. ATP is consumed to form the charged E1. Specificity for a given chain type is achieved by the choice of a specific E2 (or E2/E3 couple). Two Ub reactants are used: one reversibly capped at its C terminus and the other blocked at its lysine site. The authors defined as the "proximal end" of a chain an unconjugated G76 residue because it serves for the attachment of a proximal Ub residue. Accordingly, the "distal end" of a chain is the unconjugated lysine residue (Fig. 2A). The Ub mutant blocked at the proximal site

(also referred to as proximally blocked Ub) is Ub-D77 because the presence of an extra amino acid after G76 impairs conjugation through the C terminus of a proximal Ub. The distally blocked Ub is obtained by mutating to cysteine or arginine the lysine involved in the formation of the specific chain type by the selected E2, so that the covalent binding of a distal Ub is impaired. For example, in the case of K48-linked chains, recombinant E2-25K, able to specifically form K48-linked chains, is used to catalyze the reaction between equal amounts of Ub-D77 and Ub-K48C mutants (Fig. 2B). Thus, diUb is formed in almost equimolar concentration with respect to the monoUb reactants. The reaction will not proceed with the formation of longer chains unless the doubly blocked diUb is conveniently deprotected. A purification step (often cation exchange chromatography) will separate the product from unreacted Ub molecules.

The use of Ub mutants has some advantages with respect to the production of Ub chains using wild-type Ub. The elongation of the chain is controlled step by step. Hence, it allows differentiating between the different positions on the chain. Thus, it is possible to apply a differential isotope labeling scheme for the different Ub monomers to obtain precise structural information on the interaction of each monomer in the chain with a protein partner [29–31]. This method also permits inserting a mutated Ub in a precise position of the chain [32]. On the other hand, producing Ub mutants is more laborious than using commercial wild-type Ub, and the introduction of mutations might have some yet undescribed effects on the properties of the chains.

Deblocking of the proximal or distal terminus is necessary to form longer chains. The yeast protease Ub hydrolase-1 (YUH1) is able to remove the D77 proximal cap, forming a proximally deblocked moiety. Treatment with ethyleneimine allows the alkylation of C48 to form an *S*-aminoethylcysteine, carrying a terminal amino group that mimics a lysine residue and reacts as distally deblocked moiety. K48-linked triUb, for example, can be prepared incubating proximally deblocked diUb with Ub-D77 in the presence of E1 and E2-25K. Similarly, K48-linked tetraUb is obtained by enzymatic ligation of two single proximally and distally deblocked diUb molecules. Appropriate use of single-capped substrates for rounds of deblocking and conjugation potentially allows the preparation of chains of any desired length. The efficiency of the next conjugation step depends on the efficiency of deblocking of the capped product, which is approximately 80–90% for the YUH1 reaction and 75–90% for the ethyleneimine reaction [27]. When a chain is conjugated with a monomer, such as in the production of triUb, a 10% stoichiometric excess of the monomer reactants helps to force complete ligation of the chain and, thus, to improve the resolution in the purification step [27]. For longer chains, the separation of the product from reactants having similar length might be a problem because the resolution obtained by cation exchange is lower. Thus, it is better to use two precursors of similar size so that the unreacted chains can more easily be separated from the products (e.g., it is better for producing Ub_{12} to ligate Ub_6 with Ub_6 or Ub_8 with Ub_4 rather than Ub_{10} with Ub_2).

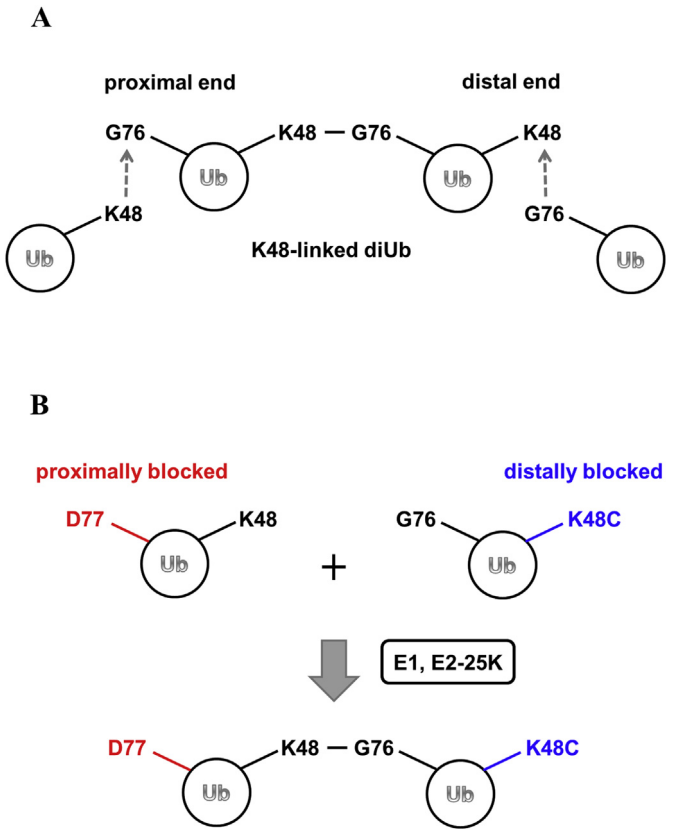

Fig.2. (A) Scheme of K48-linked diUb. Chain elongation can occur at the "proximal end" on binding of a proximal Ub or at the "distal end" on covalent conjugation of a distal Ub. (B) Production of K48-linked diUb using Ub mutants.

Synthesis of K48- and K63-linked chains

The two reactants, Ub-D77 and the specific mutant of lysine to cysteine or arginine (i.e., Ub K48C or K48R), are produced in *Escherichia coli* as untagged proteins [27]. The proteins are very stable and expressed in high amounts, allowing the production of 50–100 mg of purified proteins per liter of culture. Ub is easily purified thanks to its solubility in acid conditions. Perchloric acid (0.5%) is added to the cell lysate, causing most of the other proteins to precipitate, whereas Ub remains soluble in the supernatant. Further purification is achieved with cation exchange chromatography on an S-Sepharose Fast Flow resin (GE Life Sciences) run in 50 mM ammonium acetate at pH 4.5. The mutants are soluble to at least 110 mg/ml. Regarding the enzymes for the ubiquitination, a mammalian E1 (UBE1) expressed in insect cells infected with baculovirus is used in the protocol proposed by Pickart and coworkers [27,33]. Alternatively, E1 can be expressed as a recombinant protein in *E. coli*, although insolubility and proteolytic instability might occur because of the high molecular weight (~110 kDa) [34]. For K48-linked chains, the E2 enzyme E2-25K (also known as UbcH1) can be expressed as untagged protein or in a tagged GST form [27,35]. The Mms2/Ubc13 complex is used as E2 for K63-linked chains. His-tagged Mms2 (also called UBE2V2) is expressed in inclusion bodies, purified under mild denaturing conditions in 4 M urea using a nickel resin, and renatured by dialysis [36]. Ubc13 (also called UbcH13 or UBE2N) is expressed as GST tagged [37].

The experimental conditions for the ubiquitination are reported in Table 1. Dithiothreitol (DTT) is added to keep the enzymes reduced, but concentrations higher than 0.5 mM must be avoided because DTT can react with the activated E2 Ub thiol ester, thereby reducing the efficiency of the catalysis. The two Ub mutants are incubated with E1 and the specific E2 (E2-25K for K48-linked chains or Mms2 and Ubc13 for K63-linked chains). The reaction is carried out for 4 h at 37 °C, after which 5 mM DTT and 1 mM ethylenediaminetetraacetic acid (EDTA) are added. The mixture is incubated for reduction for 20 min at room temperature. After bringing the mixture to pH 4.0 with acetic acid, the precipitated E1 and E2 enzymes are eliminated by centrifugation. DiUb is separated from unreacted monomers by cation exchange in buffer (50 mM ammonium acetate, pH 4.5), 1 mM EDTA, and 5 mM DTT and eluted

Table 1
Experimental details for production of Ub chains using Ub mutants.

Chain type	Ubiquitination mixture	Reference
K48 or K63 (Pickart et al.)	*Substrate and enzymes:* 7.5 mg/ml Ub-D77, 7.5 mg/ml K48C or K48R, 0.1 μM E1, and 20 μM E2-25K for K48-linked chains or 8 μM each of Mms2 and Ubc13 for K63-linked chains *Buffer:* 250 mM Tris–HCl (pH 8.0), 2.5 mM ATP, 25 mM $MgCl_2$, 0.5 mM DTT, 50 mM creatine phosphate, 3 U/ml inorganic pyrophosphatase, and 3 U/ml creatine phosphokinase (ATP regenerating system)	[27]
K11 (Castañeda et al.)	*Substrate and enzymes:* K11R–K63R Ub, K63R-D77 Ub, UbE2S *Buffer:* as in Ref. [27]	[30]
K6 (Edelmann et al.)	*Substrate and enzymes:* 25 mg Ub K48R 2.9 mM, 0.1 μM E1, 0.56 μM UbE2L3, 11.1 μM NleL *Buffer:* 10 mM ATP and 10 mM $MgCl_2$ in 40 mM Tris–HCl (pH 7.5) and 0.6 mM DTT *Total volume:* 1 ml	[48]

with a 0–0.6 M NaCl gradient. A slightly higher pH value (5.2) was used for the purification of K63-linked di- and triUb in a more recent work [38]. Because polyUb chains tend to precipitate when left at pH 4.5, a quick dialysis in the final buffer is recommended. Ub chains are also rather sticky, and the unspecific absorption to surfaces reduces the final yield. Therefore, it is suggested to reuse columns, avoid glass tubes, and maximize protein concentrations to increase recovery [27]. Recently, a slightly different protocol was described for K63-linked chains in which tris(2-carboxyethyl) phosphine was used instead of DTT and no ATP regenerating system was added to the mixture [39], but the ATP concentration was increased to 15 mM.

The doubly capped diUb must be unblocked prior to chain elongation [27]. Concentrations of 16 μg/ml YUH1 [40], the enzyme used to cleave D77, are used for 1 h on half of the doubly blocked diUb to form tetraUb. The proximally unblocked diUb is then purified by anion exchange on a Q-Sepharose Fast Flow column. The second half of the doubly blocked diUb is unblocked on the distal site on reaction with ethyleneimine. After the reaction (1 h), the alkylating agent must be dialyzed away because it can irreversibly inactivate the E1 and E2 enzymes. At this point, the two singly blocked diUbs are mixed using conditions similar to those used for the first conjugation step. Pure tetraUb is separated by cation exchange [27] or size exclusion chromatography [41]. When the final length is reached, the distal C48 can be alkylated with ethyleneimine or iodoacetamide to reduce the risk of disulfide bond formation and precipitation [27]. A similar protocol for the production of K48-linked tetraUb is described in another article [42].

Synthesis of K11-linked chains

Fushman and coworkers reported a structural study on K11-linked diUb [30] based on two different strategies of sample production: one involving the use of Ub mutants and the other one with no mutations for comparison (see "Controlled synthesis of Ub chains using unnatural amino acids" section below). The E2 enzyme used to catalyze the formation of K11-linked chains is UbE2S, but this enzyme also leads to the formation of K63 chains in small amounts (see "Synthesis of K48- and K63-linked chains" section below) [43,44]. To circumvent this limitation, Castañeda and coworkers used two double mutants: K11R–K63R Ub for the distal moiety and K63R-D77 Ub for the proximal one (Table 1). K11R mutation is needed to obtain the distally blocked Ub (i.e., the site for attachment of a distal Ub is blocked); Ub-D77 mutation gives the proximally blocked Ub, whereas K63R mutation on both Ub mutants prevents formation of K63-linked chains (Fig. 3). This approach allowed individual selective isotope labeling of each Ub unit for nuclear magnetic resonance (NMR) studies. The authors also prepared K11-linked chains with no mutations to compare the chemical shift perturbation data of the two types of chains. They

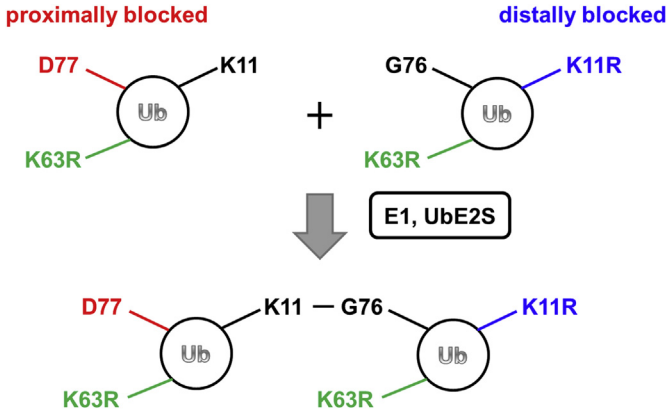

Fig.3. Strategy for the assembly of K11-linked diUb.

used genetically encoded unnatural amino acids to control Ub polymerization by UbE2S [45].

Synthesis of K6-linked chains

Komander and coworkers suggested a protocol for the assembly of K6-linked chains [31]. The method exploits the catalytic activity of NleL, a newly discovered bacterial Ub E3 ligase [46]. In the presence of E1 and UbE2L3 (also called UbcH7) [47], NleL is able to form both K6- and K48-linked chains. The authors developed two different strategies: they used as substrate either the Ub K48R, so that the enzymes will univocally form K6-linked chains, or the wild-type Ub and added to the mixture the K48-specific deubiquitylase (DUB) OTUB1 [48] (Fig. 4A and B). The reaction with Ub K48R differs from the others described previously because the mutation is introduced not to block the elongation of the K6-linked chain but rather to impair the formation of unwanted K48-linked chains.

When Ub K48R is used, the reaction is carried out at 37 °C for 3–4 h using Ub K48R, E1, UbE2L3, and NleL (detailed conditions shown in Table 1). The solution is acidified with 3 μl of 4 M HCl. After centrifugation, the Ub chains are purified by size exclusion chromatography on a Superdex 75 column (GE Healthcare) in 50 mM Tris–HCl at pH 7.6. This protocol allows production of di-, tri-, and tetraUb [31]. Specific procedures were set up to obtain K6-linked triUb samples selectively labeled on a specific Ub moiety for NMR experiments. To obtain a K6 triUb with the middle Ub labeled, diUb is first assembled using a 1:1 molar ratio of ^{13}C,^{15}N-labeled Ub K48R and unlabeled Ub K48R harboring a noncleavable C-terminal histidine tag. This construct lacks G76 to prevent proteolysis of the histidine tag during expression in *E. coli*. The diUb formed by labeled Ub (distal) and unlabeled Ub (proximal) is purified by Ni-

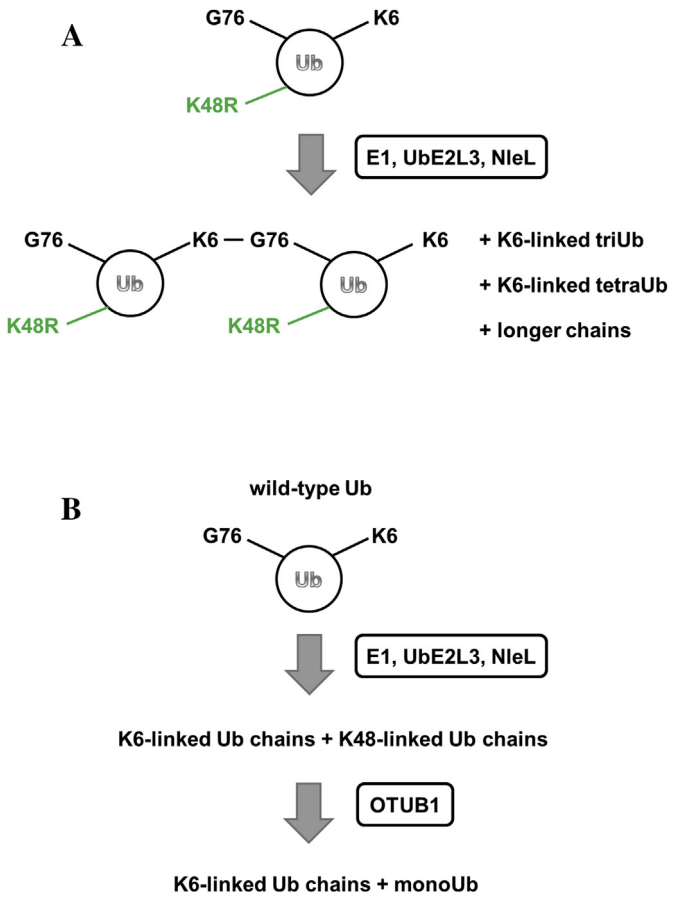

Fig.4. Strategy for the production of K6-linked chains using Ub mutants (A) or wild-type Ub and OTUB1 (B) to cleave unwanted K48-linked chains.

NTA affinity chromatography and reacted with unlabeled Ub K48R K6R, which can ligate to the diUb through its C terminus. The triUb product is purified by size exclusion chromatography.

Production of polyUb chains using wild-type Ub

A different approach for polyUb preparation is the enzymatic synthesis of chains of distinct length using wild-type Ub as substrate. This approach was developed during the early 1990s by the group of Pickart [49,50], who prepared K48-linked di- and tetraUb for crystallographic studies starting from wild-type Ub.

More recently, Komander's group produced K48- and K63-linked Ub chains using wild-type Ub [51]. The same group developed a protocol for the production of K11-linked chains [44,52]. Production of K11-, K48-, and K63-linked triUb was independently reported by Bosanac and coworkers [53]. Later on, Hymowitz, Dueber, and coworkers published a detailed methodological article exploiting the same approach [43]. The method is based on the formation of unanchored Ub chains by E1 and a specific E2 enzyme. After a few hours at 37 °C, the reaction leads to formation of a mixture of chains of different lengths. Their separation, therefore, is a crucial step of this protocol. The most common approach to perform the separation is cation exchange chromatography [43,51]. This method has the advantage of producing chains of different size in a single reaction. This can be exploited, for example, to study the affinity of Ub binding proteins with chains of increasing length. Another advantage is that, because it does not require recombinant Ub, the relatively cheap commercial bovine Ub can be conjugated to obtain milligrams of polyUb chains. Two main limits are that no selective isotope labeling of a particular position in the chain is possible because the reagents are indistinguishable Ub monomers and that chains longer than tetraUb might be difficult to be separated by cation exchange or size exclusion chromatography. This method requires the use of an E2 enzyme (or E2/E3 couple) specific for a target chain type. New protocols in recent publications describe the use of a DUB to cleave unwanted chain types formed as secondary products, thereby enabling the production of milligrams of K29- and K33-linked chains [54–56].

Synthesis of K48- and K63-linked chains

In Komander's approach [51], wild-type Ub is produced in *E. coli* and purified similarly to previous protocols for Ub mutants [27]. E1 is produced according to Trempe and coworkers [57], whereas the E2 used for K48-linked chains, Cdc34, which works similarly to E2-25K, and the E2 used for K63-linked chains, Ubc13/Uev1A, are purified according to Zhang and coworkers [58]. Lys63-linked Ub chains are produced using wild-type Ub, E1, Ubc13, and Uev1A, also called UBE2V1 (details shown in Table 2). Lys48-linked Ub chains are produced with the same protocol but using Ccd34 instead of Ubc13/Uev1A (Table 2). The ubiquitination reaction is stopped after 3 h by 20-fold dilution with 50 mM ammonium acetate (pH 4.5). The mixture of different chains is purified using a MonoS cation exchange chromatographic column (GE Life Sciences). Chains of different lengths can be separated applying a shallow gradient of 0–0.6 M NaCl in 50 mM ammonium acetate (pH 4.5). This method allows the separation of chains up to pentaUb.

A different approach set up for the production of di-, tri-, and tetraUb was reported independently [53] and, in more details, by researchers at Genentech [43]. Cdc34 and Ubc13-Uev1A are used for K48- and K63-linkages, respectively [43]. In these protocols, the enzymes are all produced in *E. coli* as affinity-tagged proteins. Wild-type Ub is mixed with E1 and Cdc34 or Ubc13-Uev1A for K48- or K63-linkages, respectively (Table 2). No ATP regenerating system is added, although the authors commented that this is compatible with the reaction, as reported in a similar procedure for the production of K11 chains [59]. The reaction mixture is incubated overnight at 37 °C. The amounts of the enzymes are chosen so that a certain percentage of Ub (>30%) would remain unreacted to avoid the risk of cyclization of Ub polymers [60]. Unincorporated Ub can be recovered and reused anyway. As a general suggestion, the authors advised paying particular attention when preparing samples for sodium dodecyl sulfate–polyacrylamide gel electrophoresis to check ubiquitination: samples should not be heated prior to gel loading in order to limit formation of high molecular weight laddering artifacts [43]. In addition, a nonreducing sample buffer should be used and samples should be mixed with loading buffer just prior to running the gel. The reaction is stopped by the addition of a 20-fold excess of 50 mM ammonium acetate and 100 mM NaCl at pH 4.5. Unreacted mono-, di-, tri-, and tetraUb species are separated by cation exchange chromatography on a MonoS column with a 0–0.6 M NaCl gradient. To obtain higher amounts of tetra- or hexaUb, it is possible to pool the diUb fraction from a first ubiquitination step, dialyze it in the ubiquitination buffer, and use it to replace monomeric Ub as the starting material. Separation by size exclusion chromatography is also used for final purification of the distinct polyUb chain lengths, for example, using a HiLoad Superdex 75 column (GE Life Sciences).

Synthesis of K11-linked chains

K11-linked chains are produced from wild-type Ub in a similar way. In Dong and coworkers' study, a truncated form of UbE2S, UbE2SΔC (M1-G156), is used as E2 (details shown in Table 2) [43].

Table 2
Experimental details for production of Ub chains using wild-type Ub.

Chain type	Ubiquitination mixture	Reference
K48 or K63 (Komander et al.)	*Substrate and enzymes for K63-linked chains:* 1.4 mM wild-type Ub, 1 μM E1, 8 μM Ubc13, 8 μM Uev1A (also called UBE2V1) *Substrate and enzymes for K48-linked chains:* same protocol as for K63-linked chains but using 2.8 mM wild-type Ub and 25 μM Ccd34 instead of Ubc13/Uev1A *Buffer:* 40 mM Tris–HCl (pH 7.5), 10 mM ATP, 10 mM $MgCl_2$, 0.6 mM DTT	[51]
K48 or K63 (Dong et al.)	*Substrate and enzymes for K63-linked chains:* 50 mg of wild-type Ub 1.0–1.5 mM, 0.5 μM E1, 2.5–5.0 μM Ubc13-Uev1A *Substrate and enzymes for K48-linked chains:* same protocol as for K63-linked chains but using 5–10 μM Cdc34 instead of Ubc13-Uev1A *Buffer:* 50 mM Tris–HCl (pH 8.0), 10 mM ATP, 4–10 mM $MgCl_2$, and 0.6 mM DTT	[43]
K11 (Dong et al.)	Same as K48 and K63 chains but using 30–50 μM UbE2SΔC (M1-G156) as E2	[43]
K11 (Bremm et al.)	*Substrate and enzymes:* 1.5 mM wild-type Ub, 250 nM E1, 4.8 μM UbE2SΔC (last 25 aa truncated) *Buffer:* 40 mM Tris–HCl (pH 7.5), 10 mM ATP, 10 mM $MgCl_2$, and 0.6 mM DTT *Total volume:* 1 ml *For longer chains:* 2.9 mM wild-type Ub, 250 nM E1, 4.8 μM UbE2S UBD; 400 nM AMSH added after 1.5 h	[44]
K6 (Hospenthal et al.)	*Substrate and enzymes:* 25 mg of wild-type Ub, 2.91 mM, 0.1 μM E1, 0.56 μM UbE2L3, 11.1 μM NleL; OTUB1 added after 3 h *Buffer:* 10 mM ATP and 10 mM $MgCl_2$ in 40 mM Tris–HCl (pH 7.5) and 0.6 mM DTT *Total volume:* 1 ml	[31]
K33 (Kristariyanto et al.)	*Substrate and enzymes:* 25 mg of wild-type Ub, 500 nM E1, 9 μM E2 UBE2D1 (UbcH5a), 6.3 μM AREL1; then added 20 μM Cezanne EK (Cezanne E287K/E288K mutant), 5 μM OTUB1, and 5 mM DTT *Buffer:* 50 mM Tris–HCl (pH 7.5), 10 mM ATP, 10 mM $MgCl_2$, and 0.6 mM DTT *Total volume:* 1.5 ml	[54]
K33 (Michel et al.)	*Substrate and enzymes:* 3 mM wild-type Ub, 1 μM E1, 10 μM E2 UBE2L3 (UbcH7), 36 μM AREL1; then treated with 1 μM OTUB1*, 1 μM AMSH*, and 400 nM Cezanne *Buffer:* 40 mM Tris (pH 8.5), 10 mM ATP, 10 mM $MgCl_2$, 100 mM NaCl, 0.6 mM DTT, and 10% (v/v) glycerol	[56]
K29 (Kristariyanto et al.)	*Substrate and enzymes:* 25 mg of wild-type Ub, 644 nM E1, 9.5 μM UBE2D3, 3 μM UBE3C; then added 2 μM vOTU and 5 mM DTT *Buffer:* 10 mM ATP, 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), and 0.6 mM DTT *Total volume:* 1.5 ml	[55]
K29 (Michel et al.)	*Substrate and enzymes:* 3 mM wild-type Ub, 1 μM E1, 10 μM UBE2L3 (UbcH7), 32 μM His-tagged SUMO UBE3C (aa 693–1083) *Buffer:* 10 mM ATP, 10 mM $MgCl_2$, 40 mM Tris (pH 8.5), 100 mM NaCl, 0.6 mM DTT, and 10% (v/v) glycerol	[56]

The C-terminus truncated version of UbE2 permitted increasing the formation of chains having intermediate size, as demonstrated previously by Komander and coworkers [44]. However, a specific problem regarding K11-linked chains is that the fidelity of the E2 enzyme used is not optimal. Thus, there is the possibility of contemporary formation of different chain types (i.e., K63 chains) in lower amounts [43]. No significant difference was observed in K11 linkage fidelity for full-length UbE2S versus the UbE2SΔC (M1-G156) truncated mutant.

Komander and coworkers developed an alternative strategy for the production of K11-linked tetraUb [44,52]. The authors found an explanation for the reduced activity of wild-type UbE2S, namely that the enzyme performs autoubiquitination in *cis* on its lysine-rich C terminus. To avoid this inhibition, they exploited two approaches. One involves using a truncated version of UbE2S missing the last 25 amino acids (so-called UbE2SΔC, a slightly longer construct than the truncated form used by Dong et al. [43]). The second requires replacing the C terminus of the enzyme with the Ub-binding domain (UBD) of IsoT to produce di-, tri-, and tetraUb [44]. The UbE2S UBD fusion protein obtained is more active than UbE2S toward the formation of K11 chains. However, this new enzyme has reduced specificity, and contaminating K63-linked chains can form. A DUB specific for K63-linked chains, AMSH, is added to the reaction mixture to circumvent the problem.

In more details, the protocol for the production of diUb [44,52] is similar to the one reported by Dong and coworkers [43]; the reaction mixture includes wild-type Ub, E1, and UbE2SΔC (last 25 amino acids truncated) reacted at 37 °C for 10 h (Table 2). The addition of 50 mM DTT is an alternative to stop the reaction before dilution with 14 ml of 50 mM ammonium acetate at pH 4.5. The purification protocol requires cation exchange chromatography. For longer chains, the more efficient UbE2S UBD fusion protein is used together with the DUB AMSH, which cleaves K63 chains. Tetramers are synthesized by mixing Ub, E1, UbE2S UBD, and AMSH (Table 2). After 1.5 h at 37 °C, AMSH is added again to cleave other K63 chains eventually formed. After 3 h, 50 mM DTT is added to the reaction before further dilution with 14 ml of 50 mM ammonium acetate (pH 4.5). Lys11-linked di-, tri-, and tetraUb are purified by cation exchange using a MonoS column (GE Life Sciences). The authors also suggested using Lys11-linked diUb as input material to obtain tetraUb [44].

Synthesis of K6-linked chains

The same conditions for the enzymatic reaction described earlier ("Synthesis of K6-linked chains" section above) were used to obtain wild-type K6-linked chains, substituting Ub K48R with wild-type Ub (details shown in Table 2). After 3 h, OTUB1 was added to the mixture and incubated overnight to cleave K48-linked chains [31].

Synthesis of K33-linked chains

Two similar protocols for large-scale enzymatic assembly of K33-linked chains were recently published [54,56]. Ligation is carried out by a HECT E3 ligase, AREL1 (also known as KIAA0317) [61], which is shown to assemble K33-linked chains, together with K11- and K48-linked chains and small amounts of K63 and K6 chains [56]. Specific DUBs, such as the Cezanne and OTUB1 DUBs or their enhanced versions, are added to obtain pure K33-linked chains after formation of a mixture of different chains. Cezanne hydrolyzes K11-linked chains [62], whereas OTUB1 is active on K48 chains [48]. Alternatively, a modified version of AMSH could be used to cleave K63-linked chains [56].

Kulathu and coworkers use a reaction volume containing wild-type Ub, E1, E2 UBE2D1 (UbcH5a), and AREL1 at 30 °C for 6 h [54] (details shown in Table 2). They then add Cezanne EK (Cezanne E287K/E288K mutant), which hydrolyzes, together with K11-linked chains, also K6, K48, and K63 linkages, OTUB1 and 5 mM DTT, and

incubate overnight at 30 °C. The enzymes are precipitated with 50 mM sodium acetate, and K33-linked chains of defined lengths are purified by cation exchange using a Resource S 6-ml column (GE Life Sciences) equilibrated with 50 mM sodium acetate (pH 4.5). The chains are eluted in a gradient with 50 mM sodium acetate (pH 4.5) and 1 M NaCl.

According to Komander and coworkers, the K33-linked chains are first separated from the enzymes by acid precipitation before the addition of the DUBs [56]. Cezanne and enhanced versions of K48-specific OTUB1 (a UBE2D2–OTUB1 fusion named OTUB1*) and of a K63-specific AMSH (a STAM2–AMSH fusion named AMSH*) are used to cleave unwanted chains. In terms of protein production, His-tagged SUMO–AREL1 (aa 436–823) is expressed in Rosetta (DE3) pLacI cells and purified by affinity chromatography. The tag is removed by SUMO protease, and the enzyme is further purified by anion exchange and size exclusion chromatography. OTUB1* and AMSH* are purified by affinity chromatography, followed by 3C protease cleavage and final purification by size exclusion chromatography. K33-linked chains are assembled using wild-type Ub, E1, UBE2L3 (UbcH7), and AREL1 overnight at 37 °C (Table 2). Glycerol (10%, v/v) is used to avoid AREL1 precipitation. The enzymes are precipitated in 0.25% (v/v) perchloric acid, and the chains are buffer exchanged in 50 mM Tris (pH 7.4), 150 mM NaCl, and 4 mM DTT and treated with OTUB1*, AMSH*, and Cezanne for 1 h at 37 °C. The DUBs are then acid precipitated and the chains equilibrated in 50 mM sodium acetate (pH 4.5) and 5% (v/v) glycerol using a PD10 desalting column (GE Life Sciences). Cation exchange chromatography on a MonoS column (GE Life Sciences) is used for the purification of K33-linked chains. A quantitative mass spectrometry method (AQUA) [63] is used to assess the purity of the product [56]. The same technique has been used previously also for the assessment of the K11-, K48-, and K63-linked chains [43].

Synthesis of K29-linked chains

Very recently, the same authors also independently described similar protocols for the enzymatic production of K29-linked chains [55,56]. They used another HECT E3 ligase, named UBE3C (or HECTH2), able to form K29- and K48-linked chains [64]. Unwanted chains were cleaved by a combination of Cezanne, OTUB1*, and AMSH* [56] or by the viral DUB vOTU [55].

Kulathu and coworkers noticed that UBE3C is also auto-ubiquitylated in the reaction mixture, so the use of vOTU is important to release free Ub species [55]. vOTU is able to cleave all chain types except M1, K27, and K29 [65]. Proteins are expressed as GST fusion and purified by affinity chromatography, followed by tag removal by C3 protease. GST UBE3C (aa 641–1083) is expressed in insect cells using a baculovirus expression system. The assembly of large-scale K29-linked chains is performed using wild-type Ub, E1, UBE2D3, and UBE3C at 30 °C overnight [55] (experimental conditions in Table 2). vOTU and 5 mM DTT are added to the reaction mixture and incubated further at 30 °C overnight. Purification is identical to the one described for K33-linked chains.

Komander and coworkers use wild-type Ub, E1, UBE2L3 (UbcH7), His-tagged SUMO UBE3C (aa 693–1083) for an overnight reaction at 37 °C [56]. His-tagged SUMO UBE3C is expressed in Rosetta 2 (DE3) pLacI cells as AREL1, and the protein is purified by affinity and size exclusion chromatography.

Controlled synthesis of Ub chains using unnatural amino acids

Fushman's and Cropp's groups developed a method to produce K11-linked diUb using a Ub substrate with modified lysines carrying genetically encoded removable protecting groups [45]. The method was first applied to K48-linked chains and later used to produce K11-linked diUb [30]. A pyrrolysyl-tRNA-synthetase/tRNAPyl pair is used to add to the genetic code of *E. coli* a lysine analogue in which the amino acid is protected with a *tert*-butyloxycarbonyl (Boc) group [66]. For the production of K48-linked chains, a recombinant Ub containing a K48Lys(Boc) mutation is produced in high yields and purity. This protein can be used, similarly to Ub K48C, as a distally blocked Ub. This construct is then reacted with a Ub mutant that is blocked on the proximal site (Ub-D77 or $Ub_{1–74}$, lacking the last two glycines) in the presence of E1 and E2-25K. K48-linked diUb is purified by size exclusion chromatography, and the Boc group is then cleaved by treatment with 2% trifluoroacetic acid. The dimer is equilibrated in the original buffer. This method could be used for another step of polymerization with Ub K48Lys(Boc) to form K48-linked triUb (Fig. 5). The synthesis of fully natural K11-linked diUb was not described in detail, but it is likely to be obtained on reaction of K11Lys(Boc) and Ub-D77 in the presence of E1 and UbE2S, with subsequent cleavage of the D77 residue by YUH1 [30].

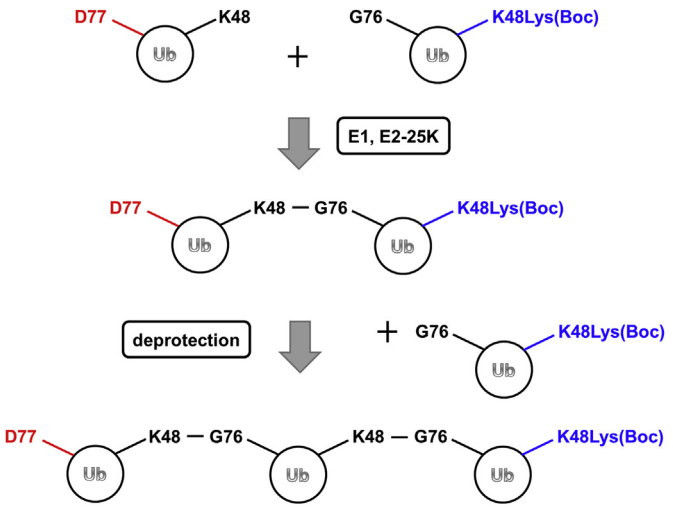

Fig.5. Approach for the synthesis of K48-linked triUb using unnatural amino acids.

Production of linear chains

The enzymes responsible for the formation of linear chains in nature have been discovered and characterized (LUBAC for linear ubiquitin chain assembly complex) [67], but the production of milligrams of chains is much easier using a suitable plasmid for each chain length required. Linear chains differ from the other chain types because the covalent bond linking two Ub units is a proper peptide bond, in that it connects the C-terminal glycine of one Ub with the N-terminal methionine of the next one. For this reason, linear chains can be easily prepared as a single polypeptide construct from a plasmid coding for the wanted number of Ub moieties. In 2008, Komander and coworkers constructed plasmids for the expression of linear di-, tri-, and tetraUb [32]. Dong and coworkers also reported a protocol for the production of linear chains [43]. The synthetic genes are optimized for efficient cloning and expression in *E. coli*. The plasmids all contain a thrombin-cleavable N-terminal His tag to facilitate purification. A second step of size exclusion chromatography is applied to obtain pure products.

Conclusions

We have reviewed here the most commonly used protocols for the production of polyUb chains of different lengths and linkages.

As is clear from the above, several different approaches are currently available, providing some flexibility of choice within their advantages and limitations. Nevertheless, the production of high quantities of highly pure material remains a laborious task that needs to be worked out and standardized. A clear shortfall is also the lack of a protocol for the production of K27-linked Ub chains, suggesting that more can still be done before the field is completely mature.

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